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Estimating genetic relationships among historical sources of alfalfa germplasm and selected cultivars with sequence related amplified polymorphisms

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Abstract Fifteen alfalfa populations consisting of six public cultivars and nine historically recognized sources of alfalfa germplasm in North American cultivars were examined using sequence related amplified polymorphisms (SRAPs). Three bulk DNA samples from each population were evaluated with fourteen different SRAP primer pairs. This resulted in 249 different amplicons, of which over 90% were polymorphic. A dendrogram from the analysis suggests that the public cultivars are quite diverse from all the historical sources of germplasm. The highest mean genetic similarity among the nine original sources of *Medicago* germplasm was 0.85 between PI 536535 (Peruvian) and 536536 (Indian), while the lowest (0.47) was between PI 560333 (*M. falcata*) and 536539 (African). The highest mean genetic similarity among the nine original sources of *Medicago* germplasm and the public alfalfa cultivars was 0.78 between PI 536532 (Ladak) and Vernal, while the lowest (0.59) was between PI 536539 (African) and Oneida. Relationships based on SRAP analysis appear to generally concur with expected relationships based on fall dormancy. This report demonstrates that SRAPs are a promising marker system for detecting polymorphisms in alfalfa.

Keywords Genetic diversity · Medicago · SRAP

Abbreviations
PI U.S. National Plant Germplasm System Plant Introduction

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Introduction

Alfalfa (*Medicago sativa* L.) is the fourth most important crop in American agriculture in terms of both cultivated acreage and farm gate value (NASS), and is globally the most important forage species in temperate climates (Barnes et al., 1988). Alfalfa is a perennial crop with a tetraploid genome ($2n = 4X = 32$) and is susceptible to inbreeding depression (Busbice et al., 1968). The great majority of alfalfa cultivars are synthetic populations that have been developed from successive generations of random mating of selected clones and their progeny (Busbice et al., 1972). Alfalfa cultivars are genetically heterogeneous and commercial cultivars of alfalfa seed are composed of thousands of plants of different genotypes. It is likely that different lots of seed of the same cultivar are also genetically heterogeneous, given the random mating that occurs in seed production fields due to the use of leaf cutter bees as pollinators.

North American alfalfa cultivars are largely derived from nine historically recognized sources of *Medicago* germplasm (Barnes et al., 1977). These germplasm sources include *M. falcata* from Russia, *M. sativa* spp. 'Ladak', an introduction from India that consists largely of *M. falcata*, *Medicago* populations from African, Chilean, Flemish, Indian, Turkistan, and Peruvian introductions, and *M. varia*, which originated in Europe from crossing between *M. sativa* and *M. falcata* (Barnes et al., 1977).

Relationships among the nine historical sources of germplasm have been previously characterized using biochemical approaches or DNA polymorphisms. Isozymes have been used to distinguish *M. falcata* from *M. sativa* (Quiros & Bauchan, 1988), but these markers are not sufficiently efficient for examining genetic diversity among a wide range of alfalfa populations. Bauchan et al. (2003) was able to distinguish the Indian source population from African, Chilean, and Peruvian source populations based on chromosome banding patterns, but this method is also not amenable for examining a large set of diverse populations. Kidwell et al. (1994) used restriction fragment length polymorphisms (RFLPs) to examine genetic diversity among the nine original sources of alfalfa germplasm. The most distinct germplasm source was *M. sativa* spp. *falcata* WISFAL, and the least genetic similarity was between *M. sativa* spp. and African *Medicago* germplasm (Kidwell et al., 1994). Although RFLPs can be useful for examining co-dominant markers, this marker system has several limitations, including the inability to resolve more than a few loci per reaction and the need for a large amount of initial template DNA relative to that required for many polymerase chain reaction (PCR) based markers.

Sequence Related Amplified Polymorphisms (SRAPs) are a relatively simple marker system that has demonstrated utility in both mapping and gene tagging in plants (Li & Quiros, 2001). The reaction is based on amplification of template DNA using forward and reverse primers that are characterized by three distinct sequence motifs: 1. The first 10–11 nucleotides at the 5′ end of each are random nucleotide sequences, followed by, 2. The sequence CCGG in the forward primer and AATT in the reverse primer, and finally, 3. Three selective nucleotides at the 3′ end of each primer. Operationally, the 10 random nucleotides at the 5′ termini of each primer may promote the amplification of arbitrary sequences, similar to what is observed with randomly amplified polymorphic DNA (RAPD) markers (Williams et al., 1990). The CCGG sequence in the forward primer is proposed to preferentially anneal to GC-rich exon sequences while the AATT sequence in the reverse primer preferentially anneals to AT-rich regions characteristic of introns and promoters (Li & Quiros, 2001). The three selective nucleotides at the 3′ termini of both primers likely confer an additional degree of selectivity similar to the 3′ termini of primers used in the selective amplification of amplified fragment length polymorphisms (AFLP) (Vos et al., 1995). Quiros et al. (2001) observed that approximately 60% of the fragments amplified from *Brassica oleracea* L. using SRAP primers shared significant similarity to reported gene sequences noted in GENBANK. SRAP markers appear to be evenly distributed among linkage groups, and this wide genome coverage has been found to facilitate the construction of linkage maps (Li & Quiros 2001).

Historically, for a variety to be accepted by the United States National Alfalfa Variety Review Board, the developers have had to present estimations for the percentage of each of the nine sources of germplasm. These estimates have been based primarily on pedigree history and morphology. The accuracy of these estimations has likely been adversely affected by the influence of environmental factors on morphological traits and the difficulty in determining pedigrees due to random mating. The objective of this experiment was to use SRAP markers to estimate genetic relationships among the nine historically sources of *Medicago* germplasm and selected public alfalfa cultivars. This is the first report on the use of SRAP markers to examine genetic diversity in alfalfa.

Materials and methods

Plant materials

Fifteen different populations were examined, including nine National Plant Germplasm System (NPGS) Plant Introduction accessions (Melton et al., 1990) representing historically recognized *Medicago* germplasm sources and six cultivars: The Plant Introduction accessions were PI 536532 (Ladak), PI 536533 (*M. varia*), PI 536534 (Chilean), PI 536535 (Peruvian), PI 536536 (Indian), PI 536537 (Turkish), PI 536538 (Flemish), PI 536539 (African), and PI 560333 (*M. sativa* spp.

Table 1 Sequences of forward and reverse SRAP primers used in this study

Forward primers	Reverse primers
Me2, 5′-TGAGTCCAAACCGGAGC-3′	Em1, 5′-GACTGCGTACGAATTAAT-3′
Me4, 5′-TGAGTCCAAACCGGACC-3′	Em2, 5′-GACTGCGTACGAATTTGC-3′
F7, 5′-GTAGCACAAGCCGGAGC-3′	Em5, 5′-GACTGCGTACGAATTAAC-3′
F9, 5′-GTAGCACAAGCCGGACC-3′	Em6, 5′-GACTGCGTACGAATTGCA-3′
F11, 5′-CGAATCTTAGCCGGATA-3′	R8, 5′-GACACCGTACGAATTGAC-3′
F12, 5′-CGAATCTTAGCCGGAGC-3′	R9, 5′-GACACCGTACGAATTTGA-3′
F13, 5′-CGAATCTTAGCCGGCAC-3′	R14, 5′-CGCACGTCCGTAATTAAC-3′
F14, 5′-CGAATCTTAGCCGGAAT-3′	R15, 5′-CGCACGTCCGTAATTCCA-3′
F16, 5′-GATCCAGTTACCGGCAC-3′	
F18, 5′-GATCCAGTTACCGGAAT-3′	

falcata WISFAL) (Bingham, 1993). The cultivars were Vernal, Fall Dormancy (FD) = 2 (Graber, 1956); Oneida (Murphy & Lowe, 1989), FD = 3; Vernema, FD = 4 (Peaden et al., 1983); Wilson, FD = 6 (Melton et al., 1989b); Malone, FD = 7 (Melton et al., 1989a) and CUF101, FD = 9 (Lehman et al., 1983).

DNA extraction

Untreated seed (not inoculated with either fungicide or *Sinorhizobium melioti*) of each population was germinated in Petri dishes on sterile filter paper. For each population, DNA was extracted from three independent bulk plant samples consisting of 20 seedlings/bulk using the Fast-DNA kit (BIO 101, Inc., Carlsbad, CA) according to manufacturer's recommendations. DNA was quantified with a fluorometer (TD-700; Turner Designs, Inc., Sunnyvale, CA), and diluted to 10 ng/ul for use in SRAP reactions.

SRAP reactions

All SRAP reactions were performed in 25 μl volume containing 50 ng DNA; 200 μM each dNTP; 1.5 mM $MgCl_2$; 2.5 units Amplitaq Gold DNA Polymerase (Applied Biosystems); 2.5 μl 10x Amplitaq Gold Buffer, and 37.5 ng of both forward and reverse primers. The thermalcycling profile for all reactions was: 95 °C for 10 min, followed by 5 cycles of 94 °C for 1 min, 35 °C for 1 min, 72 °C for 1 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. 15 μl of each reaction was electrophoresed on 15% PAGE-TBE gels (100 V for 4 h). Gels were stained for 30 min in EtBr and amplicons were visualized with μV light. The nucleotide sequences of the forward and reverse primers used in this study are listed on Table 1. All DNA samples were amplified with 14 different SRAP primer pairs: F7-Em2; F7-Em5; F7-Em6; Me4-R9; Me4-Em2; F11-R15; F16-R14; F9-Em2; Me2-R9; F13-R8; Me4-R14; F14-R9; F18-Em1, and F12-R9.

Data analysis

Gel images were scored for the presence or absence of polymorphic and monomorphic amplicons using Phoretix 1-D Pro Gel Analysis software (Nonlinear, Inc. Durham, NC). All amplicons having molecular weights greater than 100 bp were included in the AFLP analysis. It was assumed that amplicons of the same molecular weight in different bulks amplified by the same primer pair were identical.

Genetic similarities (S) were calculated for each pair-wise comparison between bulks by the method of Nei and Li (1979). The genetic similarity (S) between two samples, A and B, is calculated as follows: $S_{AB} = 2N_{AB}/(N_A + N_B)$, where N_{AB} = number of amplicons shared between A and B, N_A = number of amplicons present in A, and N_B = number of amplicons present in B. Cluster analysis was performed on the relationship matrices using the unweighted pair group method arithmetic average (UPGMA) (Avise, 1994) and the relationships were graphically presented as a dendrogram. A bootstrap resampling was performed to determine the robustness of the dendrogram. One thousand bootstrap replicates were obtained from the original data, and for each replicate a similarity matrix (Nei & Li, 1979) was calculated. From these 1000 matrices, confidence limits for each pairwise comparison were determined (Felsenstein, 1985).

Results

Performance of SRAP markers

All 14 SRAP primer pairs amplified products in all bulk DNA samples. A total of 249 amplicons were resolved, of which 23 (9.2%) were monomorphic and 226 were polymorphic (91.8%). The number of amplicons produced by each primer set ranged from 10–31, with an average of 17.8 amplicons/primer set. The percentage of polymorphic markers produced by each primer pair ranged from 66.7–100%.

An example of the amplification products of SRAP reactions is presented in Fig. 1. It can be seen that there is an amplicon of approximately 700 bp present in all three bulks of PI 560333 (*M. sativa* spp. *falcata* WISFAL) that is absent in all other bulk plant DNA extracts. In total, five amplicons were detected in all three bulks of PI 560333 that were not detected in any other bulks. Two different amplicons were detected in all three bulks of Wilson that were not detected in any other bulks. In the cases of PI 536532 (Ladak), PI 536535 (Peruvian), Vernal and Vernema, single amplicons were detected in all three bulks of a given population that were not detected in any other bulks.

Genetic relationships among alfalfa populations

A matrix of the mean genetic similarity for all pairwise comparisons is presented in Table 2. The highest mean genetic similarity among the nine historically recognized sources of *Medicago* germplasm was 0.85 between PI 536535 (Peruvian) and PI 536536 (Indian). The lowest mean genetic similarity among the original *Medicago* germplasm sources was 0.47 between PI 560333 (*M. sativa* spp. *falcata* WISFAL) and PI 536539 (African). The highest mean genetic similarity among the original sources of *Medicago* germplasm and the alfalfa cultivars was 0.78 between PI 536532 (Ladak) and Vernal. The lowest mean genetic similarity among the original *Medicago* germplasm sources and the alfalfa cultivars was 0.59 between PI 536539 (African) and Oneida, and between CUF101 and PI 536539 (African).

The mean genetic similarity between different bulks within a given population was fairly similar for all cultivars, ranging from a high of 0.95 for CUF101 to a low of 0.85 for Oneida (Table 2). Among the nine original sources of germplasm, the mean genetic

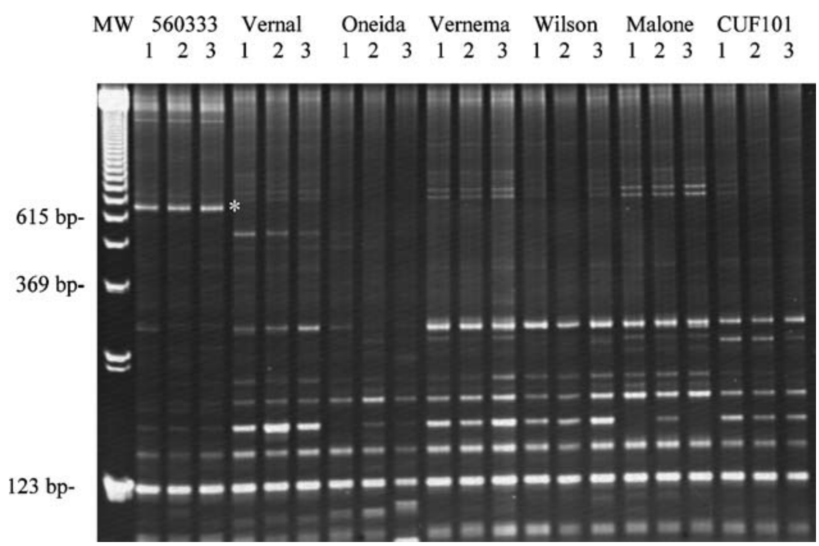

Fig. 1 Sequence related amplified polymorphisms (SRAPs) among bulked plant samples (20 plants/bulk) of selected alfalfa cultivars and PI 560333 (*M. sativa* spp. *falcata* WISFAL). SRAPs were amplified with the primer pair Me4:R9 (please refer to Table 1 for primer sequences), resolved on 15% PAGE-TBE gels and stained with Ethidium Bromide. MW = 123 bp marker.* = denotes a SRAP marker (≈700 bp) bp present in all three bulks of PI 560333 (*M. sativa* spp. *falcata* WISFAL) that is absent in all other bulk plant DNA extracts

Table 2 Mean Nei and Li Genetic Similarity among pair-wise[a] comparisons for bulk plant samples of selected alfalfa cultivars and original alfalfa populations[b] based on analysis using SRAP[c] markers

Population	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
560333 (A)	**0.90**														
Vernal (B)	0.70	**0.96**													
Oneida (C)	0.67	0.86	**0.85**												
Vernema (D)	0.63	0.82	0.80	**0.88**											
Wilson (E)	0.60	0.80	0.77	0.82	**0.89**										
Malone (F)	0.63	0.85	0.82	0.85	0.81	**0.90**									
CUF101 (G)	0.65	0.86	0.82	0.86	0.82	0.89	**0.95**								
536532 (H)	0.61	0.78	0.73	0.75	0.75	0.75	0.73	**0.91**							
536533 (I)	0.59	0.77	0.74	0.73	0.71	0.74	0.74	0.80	**0.86**						
536534 (J)	0.57	0.75	0.73	0.77	0.76	0.78	0.78	0.78	0.81	**0.88**					
536535 (K)	0.56	0.74	0.72	0.77	0.78	0.77	0.76	0.79	0.80	0.84	**0.91**				
536536 (L)	0.54	0.71	0.70	0.74	0.75	0.76	0.75	0.79	0.77	0.83	0.85	**0.86**			
536537 (M)	0.52	0.73	0.71	0.75	0.77	0.77	0.74	0.77	0.75	0.81	0.83	0.84	**0.88**		
536538 (N)	0.54	0.69	0.70	0.77	0.71	0.71	0.69	0.72	0.73	0.76	0.78	0.76	0.79	**0.78**	
536539 (O)	0.47	0.62	0.59	0.61	0.60	0.62	0.59	0.63	0.67	0.64	0.68	0.65	0.71	0.72	**0.80**

[a]Similarities in bold represent the mean of 3 pair-wise comparisons among different bulk plant samples (20 plants/bulk) of the same population. Similarities between different populations represent the mean of 9 pair-wise comparisons between bulk plant samples of each population.
[b]Letters next to each population name along the far left column correspond to the same population along the top row. 560333 = *Medicago sativa* spp. *falcata;* 536532 = Ladak; 536533 = *M. varia*; 536534 = Chilean; 536535 = Peruvian; 536536 = Indian; 536537 = Turkish; 536538 = Flemish, and 536539 = African.
[c]Every bulk plant sample was scored for the presence or absence of 249 SRAP markers.

similarity between different bulks within a given population ranged from 0.91 for both PI 536532 (Ladak) and PI 536535 (Peruvian) to a low of 0.78 for PI 536538 (Flemish).

The dendrogram presented in Fig. 2 suggests that PI 560333 (*M. sativa* spp. *falcata* WISFAL) is the most distinct of all the populations examined in this study. All of the bulk samples from alfalfa cultivars except for Oneida 3 are distributed on a separate branch from the bulks of the historically recognized germplasm sources (Fig. 2). The historic germplasm sources are distributed on the dendrogram in a manner that very closely reflects expected levels of winterhardiness and fall dormancy (Barnes et al., 1977). Among the alfalfa cultivars, Vernal (FD = 2) and Oneida (FD = 3) are grouped together closely, as are Malone (FD = 7) and CUF101 (FD = 9). Wilson appears to be the most distinct among the alfalfa cultivars examined.

Discussion

The SRAP markers proved effective at detecting polymorphisms both within and between bulked DNA samples of PI accessions representing the nine historically recognized sources of alfalfa germplasm and a select group of public cultivars. Cluster analysis (Figure 2) suggests that the alfalfa cultivars considered in this analysis have diverged considerably from all the historical sources of *Medicago* germplasm. Separation of historical source populations was generally in concordance with what is known about the winter hardiness and fall dormancy of these populations. PI 536532 (Ladak) and PI 536533 (*M. varia*) are known to be fall dormant and winter hardy, and these accessions grouped together, as did PI 536535 (Chilean), PI 536536 (Indian), and PI 536537 (Peruvian), which are all nondormant, non-winter hardy populations (Barnes et al., 1977). The least genetic similarity among the historical sources of *Medicago* germplasm was between PI 560333 (*M. sativa* spp. *falcata* WISFAL) and PI 536539 (African), the two source populations that differ most in winter hardiness and fall dormancy (Barnes et al., 1977).

The public cultivars also tended to form groups that reflected similar fall dormancies. The very dormant cultivars Oneida and Vernal grouped most closely to each other while the non-dormant cultivars Malone and CUF

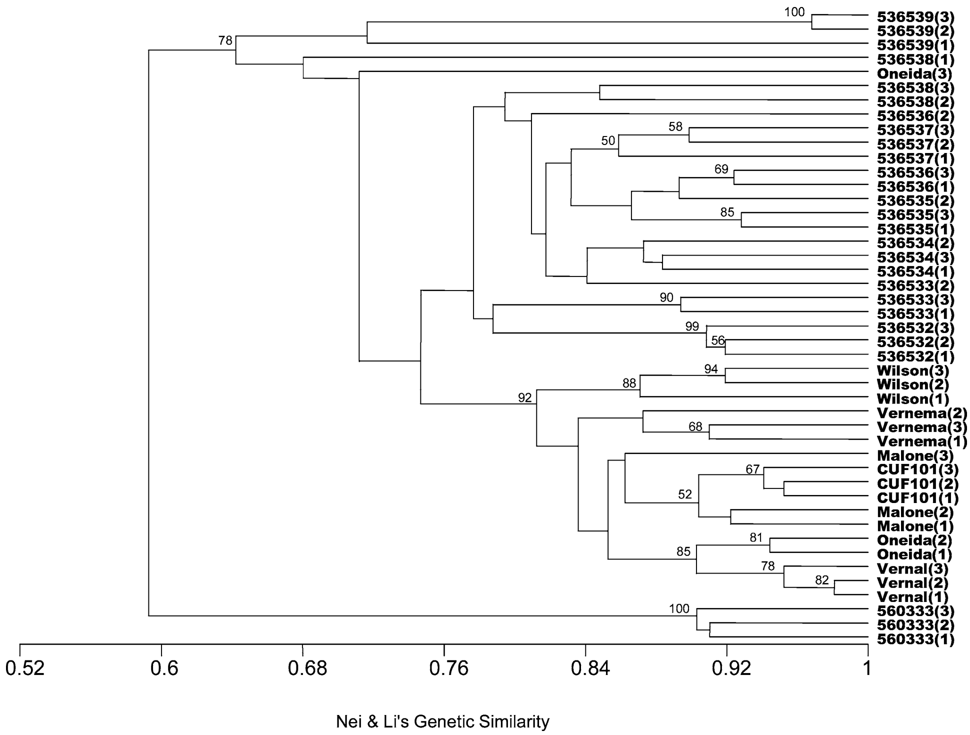

Fig. 2 Dendrogram of six alfalfa cultivars (Vernal, Oneida, Vernema, Wilson, Malone, and CUF101) and nine original sources of Medicago germplasm based on 249 SRAPs using the 'Genetic Similarity' method of Nei and Li (1979). DNA was extracted from three bulked plant samples (20 plants/bulk) of each population. Numbers above the branches represent bootstrap confidence limits ($\geq$50) for 1000 replicates

101 also formed a cluster (Fig. 2). Although these results suggest that SRAP markers may have promise for associating marker genotypes with fall dormancy, it would be necessary to examine a much larger set of cultivars to clearly determine if SRAP markers profiles could be reliably used for predicting the trait.

Not all relationships among populations based on SRAP analysis were in concordance with similarities among populations for fall dormancy. The very non-dormant CUF101 and the very dormant Oneida were the cultivars that had the lowest mean genetic similarity with very non-dormant PI 536539 (African) (Table 2). The low similarity between CUF101 and African may be in part due to changes in gene frequencies that occurred during the development of CUF101 due to selection for desirable agronomic traits lacking in African germplasm, such as spotted aphid resistance, which was most likely incorporated into CUF101 from Turkestan germplasm (Lehman et al., 1983).

Grouping among cultivars were also observed to reflect pedigree histories. Vernal and Oneida, which clustered closely together, share similar pedigrees. Oneida was developed from selections within the cultivar Iroquois, which has Vernal as a parent (Murphy & Lowe, 1968). Wilson, which commands its own robust branch on the dendrogram (Fig. 2), was developed for drought tolerance and is unique among the cultivars examined in that it contains approximately 70% contribution from Turkistan (Melton et al., 1989). Oneida was the only population for which all three bulk plant samples did not cluster together closely, with Oneida bulk 3 being positioned on the major branch containing the majority of the samples from original *Medicago* germplasm sources. Of all the cultivars, Oneida had the lowest mean genetic similarity among its three bulk samples (Table 2). Oneida was developed by the random mating of over 900 parents selected of disease resistance (Murphy & Lowe, 1989), and the heterogeneity of Oneida bulk 3 relative to the other two Oneida bulks (Figure 2) may reflect this broad parental base. SRAP markers may have been detected in Oneida bulk 3 that represent low frequency polymorphisms present in the cultivar due the large number of parental genotypes.

The ability to examine bulk plant samples would facilitate the examination of genetic relationships among alfalfa populations, which are highly heterogeneous synthetic populations resulting from multiple cycles of random mating (Busbice et al., 1972). Estimations of inter-population and intra-population genetic similarities were determined based on the analysis of DNA extracted from three independent bulk plant samples of each population, with each bulk consisting of 20 individual seedlings. Kidwell et al. (1994) demonstrated that it was more difficult to detect RFLP markers present in low frequencies with bulk plant DNA samples than with individual plants. Although the SRAP analysis presented in this report is based on the use of bulk plant samples, several relationships among populations based on SRAPs are similar to those reported based on the analysis of individual alfalfa plants with RFLPs (Kidwell et al., 1994). *M. falcata* was the most divergent of the nine original sources of germplasm and was the most divergent from African germplasm based on RFLPs (Kidwell et al., 1994), similar to what was observed for this examination with SRAPs (Table 2, Figure 2). PCR-based marker systems such as SRAPs have several inherent advantages over hybridization based markers such as RFLPs, which require that several techniques be performed, including DNA digestion, primer labeling and hybridization in order to produce reliable resolve at most a few loci per reaction (Karp & Edwards, 1998).

Several SRAPs were detected that were present in all three bulks of a single population but were not detected in any other bulk samples (Figure 1). Although these markers were present in low frequency, at least a single marker was found for PI 536532 (Ladak), PI 536535 (Peruvian), Wilson, Vernal, and Vernema that was specific to all three bulks of a given population. Five such SRAPs were identified that were specific to PI 560333 (*M. sativa* spp. *falcata* WISFAL). The relative ease demonstrated in detecting SRAPs in alfalfa suggests that it should be possible to screen more primer pairs to identify markers that are only amplified in bulk plant samples of additional germplasms and cultivars. It will be necessary to examine single plants to determine if these markers can be considered in a strict sense to be population specific.

The analysis of individual plants or additional bulk samples may at least identify SRAPs that are highly associated with specific reference populations. These SRAPs can be cloned and sequenced, and the nucleotide sequences used to develop real-time PCR assays (Livak et al., 1995). The real-time PCR assays could quantify the amount of target SRAP markers present in bulk plant samples of different populations. Given the heterogeneous nature of alfalfa cultivars, this approach might have promise for developing a series of markers that could reliably determining the relative genetic contributions of specific populations to existing and newly developed cultivars.

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